THE MECHANISM OF RADIATION EFFECTS AND THE USE OF RADIATION FOR THE PRODUCTION OF MUTATIONS WITH IMPROVED FERMENTATION

ALEXANDER HOLLAENDER

Industrial Hygiene Research Laboratory
National Institute of Health, Bethesda, Maryland

PART I

In discussions of the effects of radiation, the ultraviolet spectrum is usually divided into the so-called biologically effective region between 2000 and 3200Å and the non-active region longer than 3300Å. Studies conducted during the last few years have shown that both of these regions of the spectrum are effective. However, the energies necessary to produce recognizable effects are of a different order of magnitude. The modes of action of the various wavelengths of the ultraviolet are fundamentally different, apparently affecting various structures of the cell.

The region shorter than 3200Å is characterized by its high absorption by proteins and nucleic acids, the proteins by their low absorption band in the 2800Å region and high absorption at wavelengths shorter than 2300Å; the nucleic acids by their extremely high absorption band at 2600Å. In general, absorption spectra of biological material will show a pattern resembling protein absorption or show slight modification usually indicating nucleoproteins. It is only when the nucleic acid is concentrated in certain structures as, for instance, chromosomes, that its location can be readily recognized as has been shown by Caspersson ('36).

Considerable information in regard to the chemical characterization of the biological effect of radiation can be obtained from wavelength dependence studies of biological effects. Another method for determining what radiation will do to the cell is to extract its chemical constituents and follow their change *in vitro* by certain physical and chemical techniques. A further method is to follow changes in certain morphological structures produced by specific wavelengths in living cells. We have used all three approaches in our studies. However, we have obtained the most extensive data by the first method which I have mentioned, and rather fragmentary data by the other approaches. In studying the effects of radiation on biological materials, we have concentrated our efforts on problems which would be of direct or indirect significance to public health. This, of course, is not very difficult, since any fundamental biological approach will help us with the interpretation of the relation of disease to health.

I will discuss first a typical wavelength dependence study which we have recently completed (Hollaender and Oliphant, '44). The sensitivity of influenza virus A was determined for 8 wavelengths in the ultraviolet spectrum between 2180 and 2967Å. To get a definite measure of the sensitivity of this virus, we have irradiated a standard culture of *Escherichia coli* in the virus suspension. The

sensitivity of this organism to monochromatic radiation is well established. We found only a slight difference in the resistance of *Escherichia coli* and influenza virus. There is also very little difference in wavelength dependence of their inactivation. The absorption spectrum of influenza virus, as well as of bacteria, shows predominantly the type that you would expect from proteins mixed with a small percentage of nucleoproteins, whereas the inactivation spectra resemble more closely the pure nucleic acid absorption spectrum.

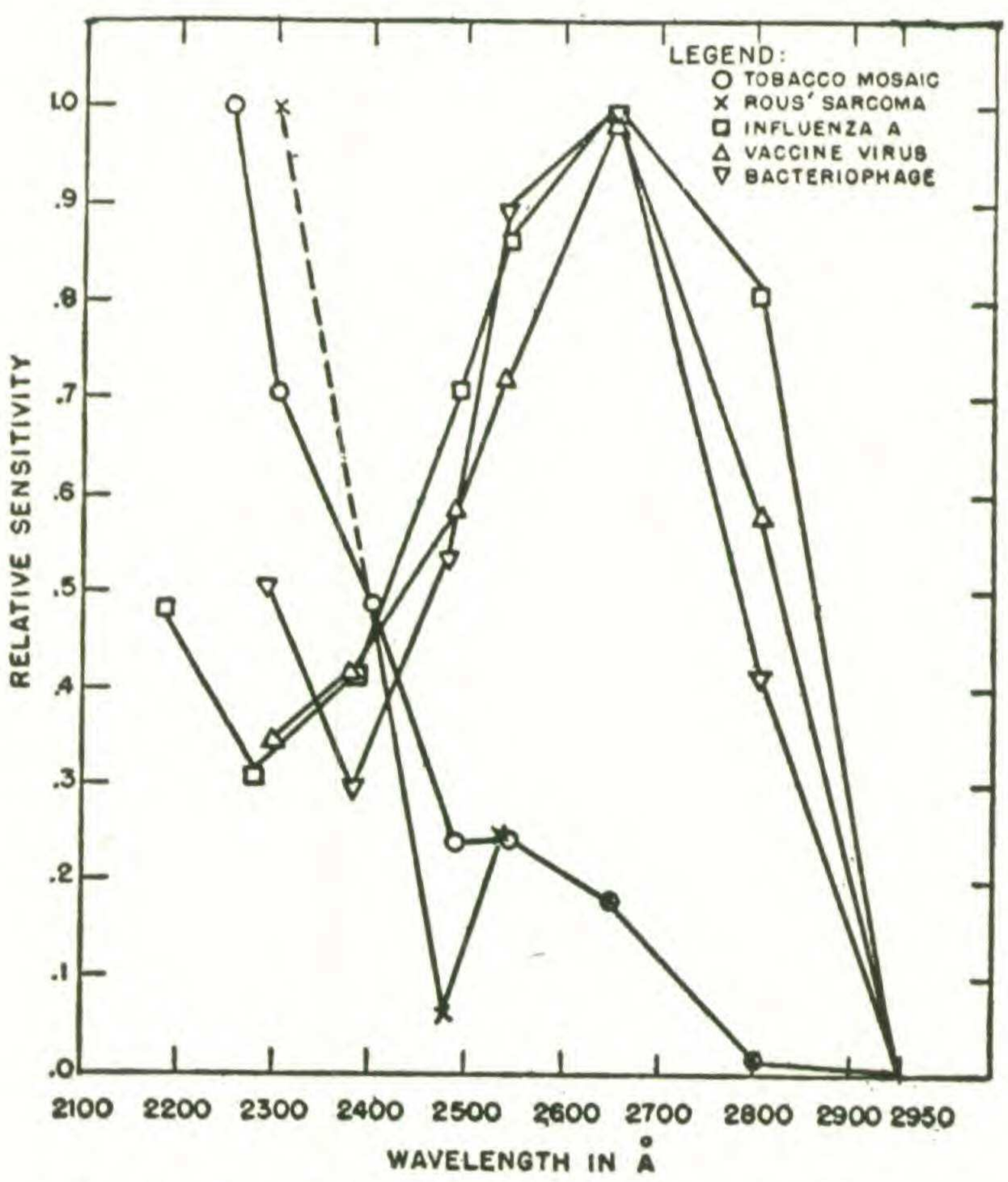

Fig. 1. Plot of the relative sensitivity against the wavelength for tobacco mosaic, Rous' sarcoma, influenza A, vaccine virus and bacteriophage, taking the energy at the wavelength which is most effective as 1 and dividing by the less effective energies. (For references see Table I, Hollaender and Oliphant, '44.)

Do all the viruses behave the same way? There are available activity spectra for five viruses: three of these have a maximum of sensitivity at 2650Å and decreasing sensitivity at shorter or longer wavelengths, and two which have a high sensitivity at 2300Å and considerably lower sensitivity in the 2600Å range. Influenza A, vaccine virus, and bacteriophage belong to the first group, and the viruses of typical tobacco mosaic and of Rous' sarcoma belong to the latter group. I mentioned before that the absorption spectra of viruses resemble more closely protein spectra with a small band typical for nucleic acids. There seems to be little doubt that, on the basis of these findings, the nucleic-acid part of the influenza, vaccine virus, and bacteriophage is the most sensitive part of these

particles, and that the protein part of tobacco mosaic and Rous' sarcoma virus is the least resistant one.

Another point which should be emphasized is that the nucleic acid of influenza, vaccine virus, and bacteriophage has been reported to be desoxypentose and the nucleic acid of tobacco mosaic and Rous' sarcoma is predominantly pentose. (For details of this study see Hollaender and Oliphant, '44. See later discussion of irradiation of nucleic acids *in vitro*.)

TABLE I

REACTIONS WITH HIGHEST SENSITIVITY AT 2650Å

Inactivation of viruses and virus-like agents	Influenza (Hollaender & Oliphant, '44) Vaccine (Rivers & Gates, '28) Bacteriophage (*St. aureus*) (Gates, '34)	
Killing of microorganisms	Bacteria: All types—Pathogenic and Saprophytes	For review see Hollaender, '42
	Fungi: Yeasts (Oster, '34); *Trichophyton* (Hollaender & Emmons, '39); *Aspergillus terreus* (Hollaender, Raper & Coghill, '45)	
Mutation production	Fungi: *Trichophyton* (Emmons & Hollaender, '39); *Neurospora* (Hollaender, Sansome, Zimmer & Demerec, '45); *Aspergillus terreus* (Raper, Coghill & Hollaender, '45); *Penicillium notatum* (Hollaender & Zimmer, '45)	
	Higher Organisms: *Sphaerocarpus* (Knapp, Reuss, Risse & Schreiber, '39); *Zea Mays* (Stadler & Uber, '42); *Drosophila* (MacKenzie & Muller, '40; Demerec, Hollaender, Houlahan & Bishop, '42)	
REACTIONS WITH VERY HIGH SENSITIVITY AT λ 2300Å AND A SMALL MAXIMUM AT 2800Å OR 2600Å		
Inactivation of viruses and enzymes	Tobacco mosaic (Hollaender & Duggar, '36) Rous' sarcoma (Sturm, Gates & Murphy, '32) Urease (Kubowitz & Haas, '33)	
Killing of higher organisms	*Enterobius vermicularis* (Hollaender, Jones & Jacobs, '40) *Ascaris* (Wright & MacAlister, '34)	
Parthenogenesis of *Arbacia*	*Arbacia* (Hollaender, '38)	

Table I shows a list of biological reactions for which sufficient data are available on the effect of monochromatic ultraviolet radiation to permit their being

classified and fitted into the predominantly protein or the nucleic acid pattern. It is not surprising that bacteria have a maximum sensitivity at 2650Å, since, as far as tested, they are made up of a high percentage of nucleic acids. The same applies to yeasts and fungi but the 2600Å maximum of sensitivity in many fungi is obscured by the protective absorption of pigments.

Whenever 2600Å radiation has been tested for mutation production and the conditions have been such that the radiation could penetrate readily to the nucleus, high efficiency in producing genetical changes for this wavelength has been found. Our early studies with Dr. Emmons on *Trichophyton* have now been repeated with *Neurospora* in a cooperative study with Dr. Demerec and Mrs. Sansome. The results of this study verify our findings on *Trichophyton:* 2650Å is the most efficient wavelength in producing mutations. This work on fungi, which I will discuss in the second part of my paper, has also been extended to *Penicillium notatum* and *Aspergillus terreus*.

I am sure you are acquainted with the work on higher organisms. I included the work on *Drosophila* under the 2650Å section in spite of the fact that the wavelength which is most effective on this organism is 3130Å. The probable reason for this is that the sperm has to be irradiated inside the fly and the abdominal wall prevents the 2650 wavelength from penetrating readily to the sperm. It is unfortunate that the artificial insemination technique has not proved practical.

The second part of this table shows a number of biological reactions with high sensitivity in the very short ultraviolet (<2300Å). This would indicate that the protein part of these materials is the most sensitive one. The inactivation of tobacco mosaic and Rous' sarcoma shows also a small maximum at λ 2600Å, indicating that the nucleic-acid part of these materials has a slight sensitivity in this region.

The higher organisms described in the rest of this table are surrounded by heavy protein membranes which explain their sensitivity in the short ultraviolet. Little information is available at the present time in regard to tissue cultures. Crude work has shown that this material shows its highest sensitivity at short wavelengths.

It would not be surprising, however, that careful studies which take into account the action of protective materials would bring out a fairly high sensitivity in the 2600Å region.

Summarizing, it is well to point out that the wavelength-dependence studies have given us an opportunity to obtain an indication of the chemical structure in living substance, which is most easily interfered with by radiation.

In an effort to get a better understanding of the effect of radiation on living materials, we studied some of the constituents of living cells *in vitro*. We have studied the effect of 2537Å radiation on sodium thymonucleate (Hollaender, Greenstein and Jenrette, '41) and certain serum proteins (Davis, Hollaender and Greenstein, '41). The changes most readily produced are the result of alteration in the

physical properties of the treated compounds, for example: viscosity, stream birefringence and colloid osmotic pressure. While the changes produced in the isolated compounds of the living cell or directly in the living cell are doubtless qualitatively similar, quantitatively they must appear to differ enormously. This is probably due to the difference in detectability of the two types. Changes in the isolated components must be detected by physical methods which require that a relatively large number of the molecules of the compounds under study be altered, and this, in turn, requires very large doses of radiation. The structures of the living cell, on the other hand, even though they consist of these same or similar compounds are parts of very delicately balanced and precisely adjusted units, in which changes induced in a few molecules by relatively low doses of radiation may alter radically certain detectable behavior and structural characteristics of the cell (Carlson and Hollaender, '44). Very little is known about the state of the relation of protein to nucleic acids in living cells. A search of the literature on this subject reveals that there is still considerable confusion about the exact structure of nucleo proteins (Greenstein, '44) and further work in this field is urgently needed.

The effect of 2537Å was studied (Carlson and Hollaender, '44; Kaufman, Gay and Hollaender, '44) in an effort to obtain information in regard to the mechanism of the influence of radiation on mitosis. Although this study is in its early phases, the results indicate that the early prophase is retarded most by 2537Å radiation, in contrast to X-rays where the middle and late prophases are most sensitive. The high sensitivity of chromosomes to 2537Å radiation is well demonstrated by the fact that an exposure to a total of 1500 ergs per square centimeter, either given in 1 second or spread over 1500 seconds, will produce a measurable retardation of mitosis in grasshopper neuroblasts in tissue cultures.

Up to this point, I have discussed the effects of radiation shorter than 3200Å. The action of radiation in the long ultraviolet has been more or less ignored. One reason is that most non-pigmented biological materials have very little absorption in this region; as a matter of fact, so little that our present means of taking absorption spectra are not sensitive enough to detect this absorption. This also explains why the energies necessary to produce changes in the long ultraviolet are of different order of magnitude than the ones at shorter wavelengths. For instance, we can produce very striking effects if we give bacteria 10,000 to 100,000 as much energy at 3650Å as was necessary to produce recognizable effect at 2650Å. Besides its lethal action, 2650Å will produce a delay of growth in surviving organisms; in other words, a prolongation of the "lag" phases. This prolongation of the lag will be about 50 per cent of the normal lag. The 3650Å range may increase the normal lag phase tenfold. It will also change the permeability of the cell. We have summarized these effects in Table II.

It appears that the effect produced by this wavelength is through action on the colloid structure of materials irradiated as well as in the structure of certain respiratory enzymes. The function of the long ultraviolet is important from

TABLE II

EFFECTS OF LONG ULTRAVIOLET AND NEAR VISIBLE RADIATION ON *ESCHERICHIA COLI*

	3400 to 4400Å	2180 to 2967Å
1. Shape of killing curve (log survival ratio/energy)	Threshold type	Approaching straight line
2. Energy (incident) for 50% survival ratio	Approximately 2×10^8 ergs/cm.^{2}	5×10^2 to 10^3 ergs/cm.^{2}
3. Temperature coefficient	1.7 — 2.2	1.1
4. Sublethal effects appear	Before any organisms are killed (in threshold part of killing curve)	After 60 to 90% of organisms are killed
5. Extension of retarded growth phase for 10% survival ratio	Up to 1000%	50%
6. Toxicity of certain salt solutions can be recognized	At once after irradiation	In 600 minutes at 32° C.
7. Mutation production	No mutations	Mutations produced in fungi and *Drosophila*

the ecological point of view, since this radiation is quite intense in sunlight.

In summary, the study of the response of microorganisms to ultraviolet radiation has established distinct effects which each wavelength range produces. The wavelengths which are most highly absorbed by nucleic acids (2600Å) are most efficient in producing mutations. Other wavelengths which are absorbed more generally by the cell (3650Å) show their effect in a retardation of growth and an interference with the normal respiration of the cell. Several regions of the spectrum still await an interpretation of their effects on the living cells. The field of the combination of different wavelength ranges is an especially promising one for further investigation.

PART II

The production of mutations by ultraviolet radiation follows a definite quantitative pattern. The maximum mutation rate is reached after the organisms have been exposed to certain amounts of energy. A further increase in energy tends to decrease the mutation rate from this maximum rate. In contrast to this, the increase of mutation rate with increasing energy in the X-ray region is more or less linear. These typical mutation curves have been established not only with the *Fungi Imperfecti* but have also been found with *Neurospora crassa* (Sansome, Demerec and Hollaender, '45; Hollaender, Sansome, Zimmer and Demerec, '45).

It was thought when this work was begun that it might be possible, by radiation techniques alone, to produce mutations of certain predetermined properties. Experience has shown that it is not yet possible to accomplish this. However, it has been found that the ultraviolet will produce a predominance of gene mutations while the X-rays tend to produce a predominance of chromosomal aberrations and chromosome breaks (Stadler and Uber, '42).

Most of the early work on mutation production in fungi established the mutation rate on the basis of "morphological" changes. But the fundamental reactions which cause the appearance of morphological mutations are no doubt "biochemical." Early in the war it became desirable to produce changes in certain organisms which were capable of producing urgently needed chemicals. This led us to suggest the use of radiation techniques for this purpose.

The usual tendency in all induced mutation work is to produce changes in the organisms which result in reduced activity. This is probably due to an interference with certain enzyme systems. This type of approach has been established by Beadle and Tatum ('41). The so-called "progressive mutations," i. e., mutations with improved fermentation, have only occurred occasionally. The difficulty here probably lies in the fact that several gene modifications are necessary to induce a mutation with increased yield while a "deficient" mutation may be caused by single gene changes.

The results of most fermentation processes of fungi are not alcohols, acids, etc., of high purity, but usually a mixture of more or less closely related compounds. Thus the suppression of an undesirable reaction, through interference with the enzyme system causing it, is a promising possibility. However, as can be seen below, if one interferes with one enzyme system, there is a tendency for the whole chain of systems to be disrupted, probably because of the close interrelationship of the different systems within the organism.

We will now discuss a number of studies where an attempt has been made to influence fermentation in a direction most desirable to the experimenter. Cultures which survived X-radiation and show deficiencies in development were observed as early as 1904 at a time when the early biological exploration of Roentgen's discovery was at its height (Dauphin, '04). Observations of Nadson ('25) showed that colonies of yeast on the border of irradiated areas in Petri dishes grow more profusely than the colonies protected against radiation.

An extensive study of the production of citric acid by *Aspergillus niger* as influenced by radium emanation and ultraviolet light was reported by Kresling and Stern in 1936. They observed an increase of citric acid in the cultures when grown in the presence of radon, but no increase of citric acid was observed when the cultures were grown under ultraviolet. A number of strains were isolated from the irradiated cultures. The results of these tests for acid production of these mutations are given in Table III. All of the new strains produce equal or less amounts of acid than the controls.

Early in the development of the mass production of penicillin by *Penicillium*

TABLE III

BIOCHEMICAL PROPERTIES OF RADIUM STRAINS OF *ASPERGILLUS NIGER**

Strains	Acid in grams per 100 ml fermentation solution			Sugar in grams per 100 ml solution		Dry weight of mycelium in grams
	Citric Acid	Gluconic Acid	Oxalic Acid	Used	Left over	
Control Strain #3	5.21	0.90	0.19	13.35	6.65	4.579
Radium Strain #3_1	0.18	2.91	0.00	11.66	8.34	3.822
Acid Strain #3_2	0.00	2.60	0.00	12.50	7.50	4.132
Control Strain #1	1.64	0.34	0.33	18.7	1.3	4.934
Radium Strain #1_1	0.00	0.58	0.00	15.0	5.0	4.872
Control Strain #6	11.88	1.16	0.33	18.7	1.3	3.554
Radium Strain #6_1	0.68	0.84	0.33	15.0	5.0	3.869
Radium Strain #6_2	0.00	2.33	0.20	14.0	6.0	4.761
Radium Strain #6_3	7.52	0.73	1.00	15.4	4.6	3.624

*Table taken from: Über die Wirkung von Radium- und ultravioletten Strahlen auf die Entwicklung, die biochemischen Eigenschaften und die Rassenbildung des *Aspergillus niger*. (Kresling and Stern, '36, p. 339).

TABLE IV

EXPERIMENT F3—*PENICILLIUM NOTATUM*. CULTURE 10 DAYS OLD IRRADIATED WITH λ 2650Å

	Energy per spore	Per cent survival	Number colonies isolated	Per cent mutation
Control	0	100	75	0
Run 1	3.0×10^{-3} ergs	43.0	74	1.3
2	9.4	37.4	76	1.3
3	19.5	14.7	75	2.0
4	34.1	5.4	81	13.6
5	42.9	.2	81	12.3
6	71.5	.03	80	8.8

notatum, we had an opportunity to discuss with Dr. Heatley of the Oxford group the possibility of producing mutations with increased penicillin yield by irradiating *Penicillium notatum* spores with monochromatic ultraviolet and possibly X-rays. We started this investigation in cooperation with Dr. Emmons of the National Institute of Health in 1941 and later continued it in cooperation with the Cold Spring Harbor group.

The production of the morphological mutations follows the usual pattern: high efficiency of mutation production with wavelength 2650, lower efficiency at shorter and longer wavelengths, and no mutation production at wavelengths in the 3650Å region. The mutation rate increased with increasing energy and became more or less erratic at still higher energy values. In contrast to this, the effect of X-rays on morphological mutations follows a more or less straight-line relationship. I will return to this point later on. Typical results of a single irradiation test are given in Table IV. A typical killing and a mutation curve are shown in fig. 2.

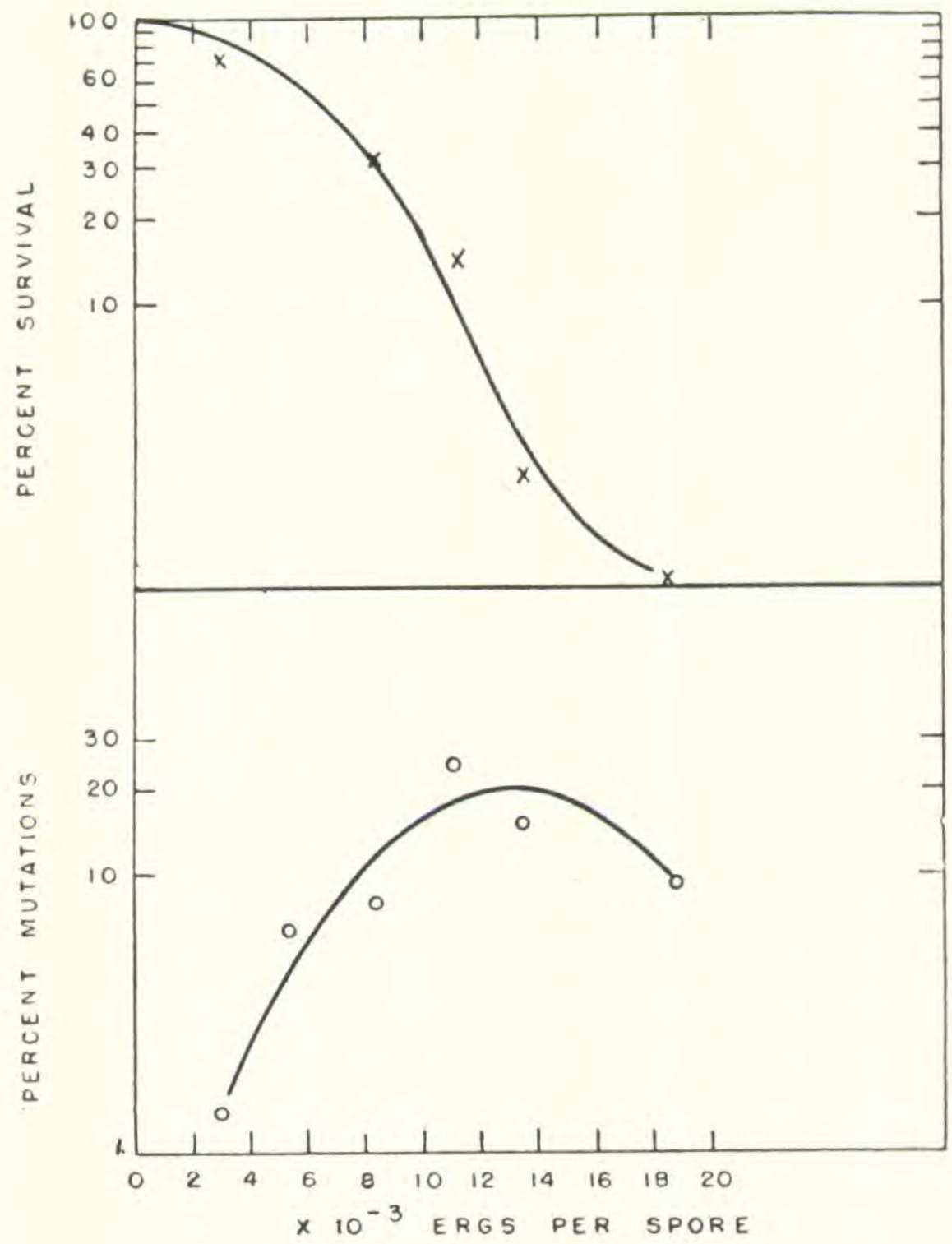

Fig. 2. *Upper graph:* Per cent survival against energy absorbed per spore for *Penicillium notatum. Lower graph:* Per cent mutation against energy. Each point on the lower graph corresponds to point in upper graph for same energy value.

No general biochemical investigation was conducted in connection with this study. However, in a collaborative study with Dr. J. W. Foster of Merck & Co.,

at Rahway, N. J., a number of irradiated and control cultures were tested for penicillin production at the Rahway laboratory. Penicillin production was determined after 2, 3, 4, and 5 days in submerged shaking cultures. The concentration was determined by the standard cup method. The results of a typical set of data are given in Table V. Typical yield distribution plots are given in fig. 3. The mean of the penicillin production for control and irradiated cultures is also given.

TABLE V

TYPICAL SET OF TESTS FOR CULTURES OF *PENICILLIUM NOTATUM* COMING FROM IRRADIATED AND CONTROL SPORES (JULY 1943)*

Culture	Oxford Units per ml		
	2 days	3 days	4 days
F_3 5.25	17	25	8
26	23	29	11
27	18	40	13
28	17	43	28
29	24	52	29
31	26	42	28
32	19	40	29
33	< 8	< 8	29
36	< 8	< 8	28
F_5 5.10	< 8	< 8	8
13	< 8	< 8	8
32	< 8	11	13
36	< 8	54	8
37	< 8	22	8
44	22	80	8
45	28	49	11
49	23	54	8
56	16	56	12
60	25	59	20
66	24	80	32
Control	33	46	17

*Tested by J. W. Foster, Merck & Co., Rahway, N. J.

The irradiated cultures show, in general, a very wide distribution of variation in the yield of penicillin with a predominance of low-yielding strains and some which practically did not produce any penicillin. However, occasionally a mutation was produced which gave an unusually high yield. Of about 200 cultures tested, two were found of this type. The distribution of yield of cultures seems to be definitely towards the lower side. It is unfortunate that the difficulty of testing *Penicillium notatum* for penicillin production makes it cumbersome to run through a large number of cultures under a variety of conditions which might bring out more clearly the interesting mutations. There seems to be little, if any, relation between morphological mutation and change in penicillin production. As a matter of fact, the normal-appearing cultures have a tendency to give the higher yields.

Another study was conducted in cooperation with Dr. Raper, Dr. Coghill and

others of the Northern Regional Laboratory (Hollaender, Raper and Coghill, '45; Raper, Coghill and Hollaender, '45). The purpose of this investigation was to attempt to produce mutations in *Aspergillus terreus* which would have increased itaconic acid production.

This organism distinguished itself by a very low sensitivity to ultraviolet radiation. However, it showed itself to be more sensitive to mutation production than any of the other organisms tested. While most of the organisms tested show their highest mutation rates with ultraviolet when 90 to 95 per cent of spores are killed, *Aspergillus terreus* shows its highest mutation rate after 25 to 40 per cent of the spores are inactivated.

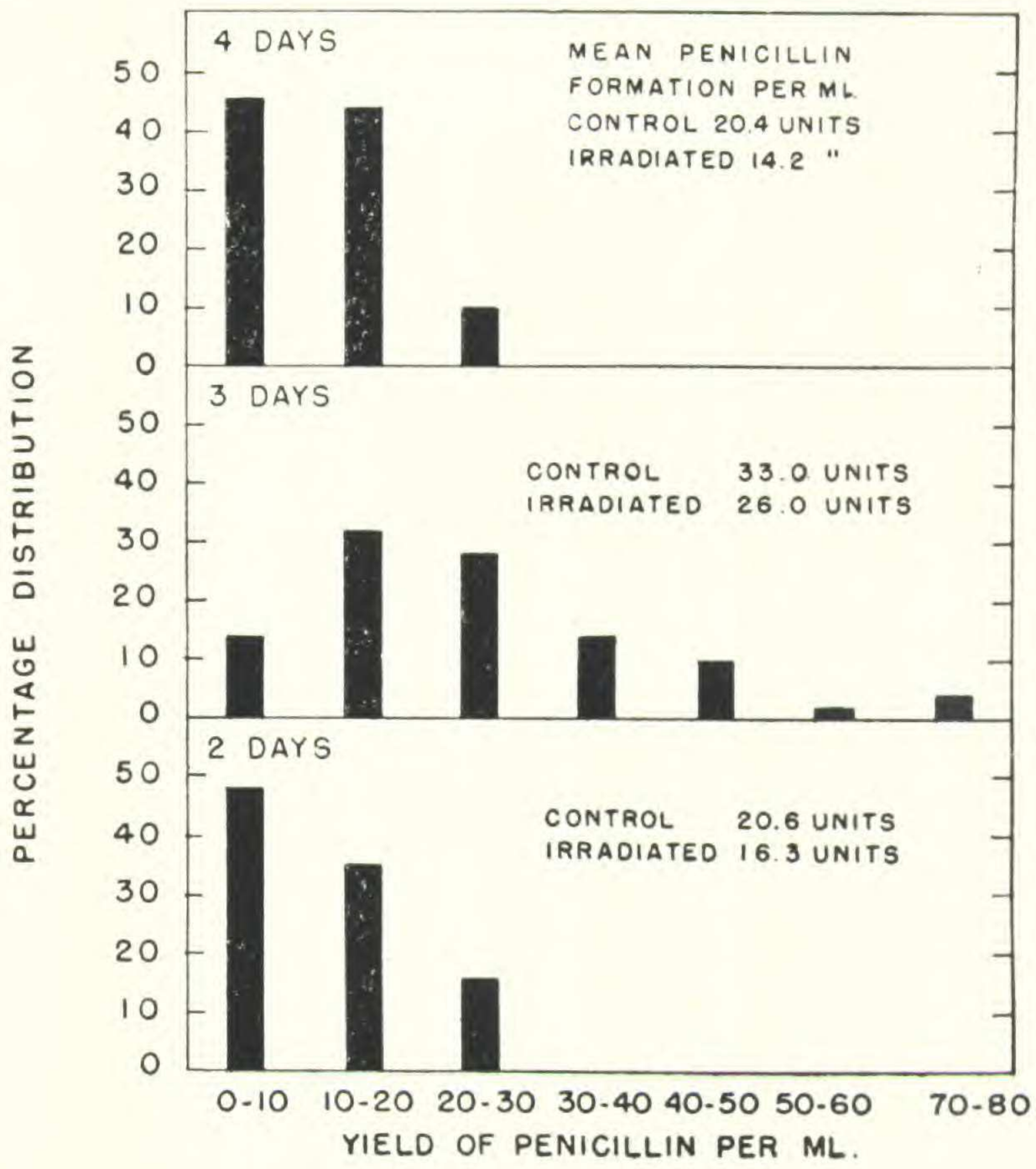

Fig. 3. Per cent distribution against yield of penicillin in Oxford Units based on tests made by Dr. J. W. Foster. Time of tests refers to days of incubation.

The morphological mutations showed wide variety in appearance. Several interesting mutations were found which showed certain deficiencies when grown in Czapek solution agar but which appeared normal on a more complete medium (malt extract agar). One of these is a thiamin-deficient mutation. When grown in Czapek solution agar it forms a thin spreading mycelium, while in malt extract agar it duplicates the normal mycelium. Another mutation appears deficient when grown on a nitrate medium, but when it is grown on a medium with ammonia or amino nitrogen the culture appears normal. A number of other deficiencies have appeared which await analysis.

In a separate study Lockwood, Raper, Moyer and Coghill ('45) investigated 217 irradiated cultures for their ability to produce itaconic acid. It was thought that it would be possible to inhibit some of the enzyme systems which would then leave the organism to ferment a higher percentage of the sugar to itaconic acid.

I am quoting from their summary:

> "Nine different types of biochemical and cultural response have been observed from 217 strains of *Aspergillus terreus* derived from irradiated conidia.
>
> "Among the 76 strains which were morphologically unchanged were 59 which appeared to be unaltered biochemically, 13 which produced more itaconic acid than the parent strain, and 4 which produced no itaconic acid.
>
> "Among the 141 strains which were obviously altered morphologically were 42 strains not apparently altered biochemically, 88 which produced little acid, and 11 which did not grow on the test medium. None of these 141 strains produced more itaconic acid than did the parent strain.
>
> "Fifteen strains produced considerable non-acidic unsaturated material.
>
> "Seventeen strains appeared to produce no acid other than itaconic."

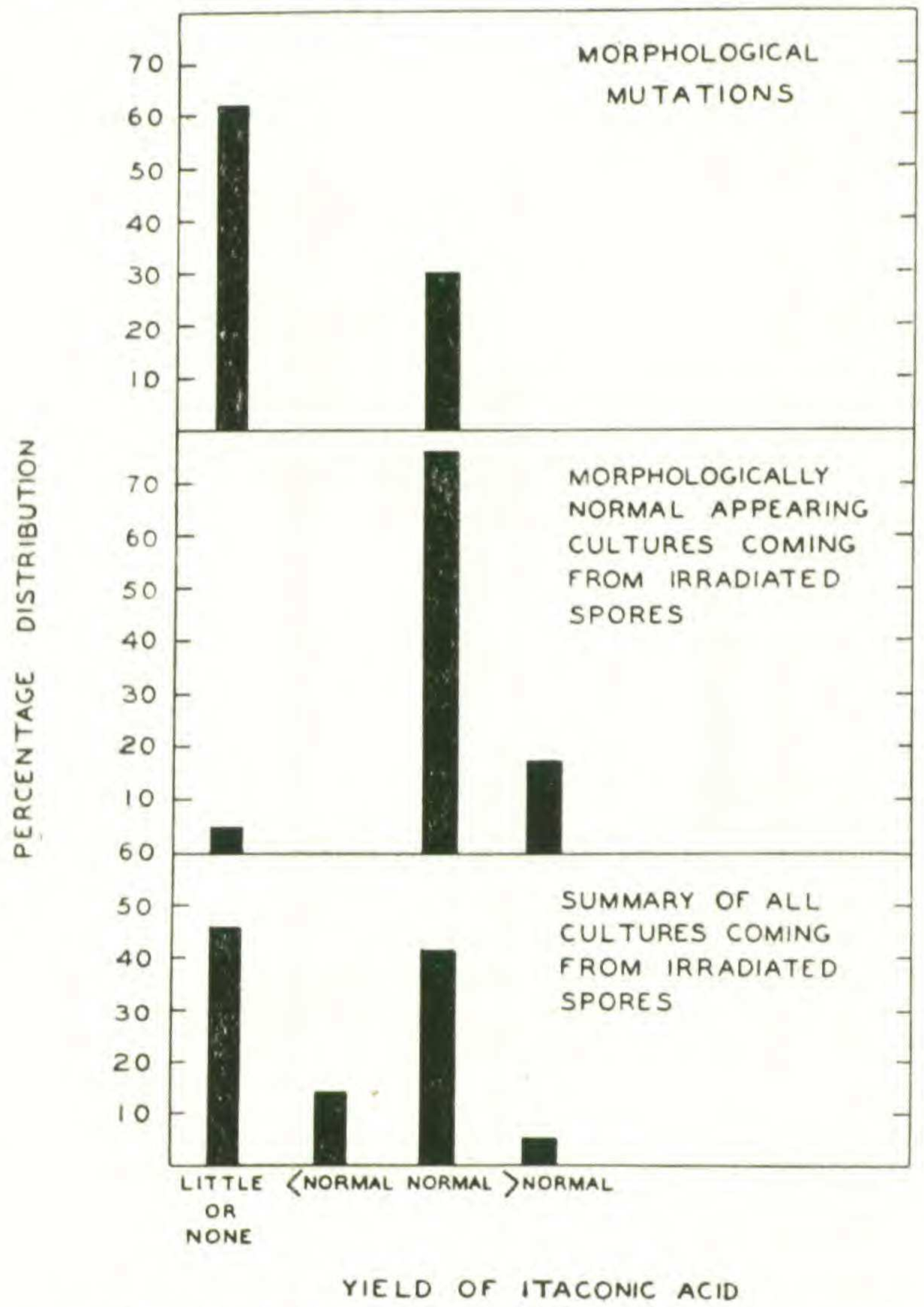

Fig. 4. Distribution of yield of itaconic acid produced by *Aspergillus terreus* mutations based on Lockwood, Raper, Meyer and Coghill ('45).

A block diagram of the percentage distribution of the tested cultures on the basis of yield of itaconic acid is given in fig. 4. The tendency of morphological

mutation to give lower yields of itaconic acid is well demonstrated.

It is not unusual to find in nature strains of *Penicillia* or *Aspergilli*, believed to represent mutations, which have different biochemical activity from the usual standard "accepted" strains; and there is good reason to expect to find in the naturally occurring strains occasionally one with more desirable fermentation properties. This type of mutation might very well have survived by natural selection. Such strains have actually been found with *Aspergillus terreus* (Raper, Coghill and Hollaender, '45). A promising investigation would be the irradiation of these new high-yielding strains and the study of the mutations produced.

If we analyze the data from these three sets of experiments, we can conclude that it is not difficult to interfere with the normal metabolism of an organism. The combination of interferences which would result in an increased production of certain chemicals can not be expected to happen often.

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